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**STUDIES ON THE PATHOGENESIS OF BIS (2-CHLOROETHYL)
SULFIDE (HD) INDUCED VESICATION IN PORCINE SKIN.**

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ABSTRACT

HD is a potent vesicant whose pathogenesis remains speculative. Previous work with the isolated perfused porcine skin flap (IPPSF) described the HD dose-response relationship. The present work includes a time-response study, probes the effects of alterations in basal perfusion parameters (glucose concentration, perfusate flow), and assesses the prostaglandin (PGE_2 , 6-keto $\text{PGF}_{1\alpha}$) profile of HD vesication. Immunohistochemistry and immunoelectron microscopy created a map of the epidermal-dermal junction epitopes in HD-induced blisters. In the time response study, 10 mg/ml HD was applied to IPPSFs and terminated at 1, 3, 5 and 8 hrs. In the perfusion parameter study, IPPSFs were perfused at 0.5, 1.5 or 3.0 ml/min and 50, 120 or 400 mg/dl glucose concentration. Viability was assessed by glucose utilization, vascular resistance, LDH release, glucose/lactate ratio, enzyme histochemistry, light and transmission electron microscopy. HD exposure was characterized by changes in nucleolar and mitochondrial structure, glucose utilization, lactate/glucose ratio, and enzyme histochemistry at 3 hrs in the absence of microvesication. At 5 hrs, microvesication occurred and signs of cytotoxicity were more severe. Changes in perfusate flow did little to modulate the HD response. Low glucose resulted in blister formation in controls, while high glucose blocked the formation of microvesicles and blisters in HD treated IPPSFs, despite the presence of severe cytotoxicity. HD induced an increase in PGE_2 flux. HD blisters showed laminin bound to the lamina lucida, type IV collagen to the lamina densa, and bullous pemphigoid antibody to the epidermis. This pinpoints the HD-induced plane of epidermal-dermal cleavage to the upper portion of the lamina lucida.

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INTRODUCTION

The mechanism of cutaneous vesication induced by the military agent sulfur mustard (HD) has eluded investigators since World War One. Progress to date has been reviewed in a 1991 monograph by Papirmeister et al. (1). Part of the problem resides in the model systems used. Humans exposed to topical HD will usually develop a fluid-filled blister after a delay of a few hours. When animals are exposed *in vivo*, some will blister; however, many do not, with the experimental end point often being histological evidence of microblister formation. With *in vitro* human and animal model systems, gross blisters never form and microblisters are the accepted end point. Part of this discrepancy must be a result of the complex pathogenesis of chemical vesication. Although *in vivo* models may produce the relevant lesions, mechanistic studies are difficult to conduct because humane considerations may preclude their use.

The isolated perfused porcine skin flap (IPPSF) is a novel *in vitro* animal model that we developed to study drug and chemical percutaneous absorption and cutaneous toxicity (2-13). The major advantage of this system is that it is a viable, full-thickness skin preparation which possesses an intact vasculature, a relatively large surface area for dosing, and control over experimental parameters and sample collection. Since pig skin is a well-accepted animal model for percutaneous absorption studies (14,15), the IPPSF should also be an excellent alternative animal model for investigating cutaneous toxicity. Studies with the HD monofunctional analogue 2-chloroethyl methyl sulfide (CEMS) (3) demonstrated that the IPPSF produced gross fluid-filled blisters after exposure to this chemical vesicant. The IPPSF would appear to be an optimal alternative animal model since its level of biological complexity is intermediate between simpler *in vitro* systems and the *in vivo* setting. The purpose of these studies is to investigate the pathogenesis of HD vesication in the IPPSF using a time-response experimental design as well as by manipulating biochemical and physiological perfusion parameters.

MATERIALS AND METHODS

Female Yorkshire weanling pigs weighing 20-30 kg were used. Skin flaps were prepared and perfused as described previously (2,5,7,9,16). The perfusion medium is a modified Krebs-Ringer bicarbonate buffer (pH 7.4, 350 mOsm/kg), containing albumin (45 g/l), and supplied with glucose (80 to 120 mg/dl) as the primary energy source. Normal perfusate flow through the skin flap is maintained at 1.5 ml/min/flap (3 to 7 ml/min/100 g), with a mean arterial pressure ranging from 30 to 70 mmHg. Samples were taken of the arterial medium and the venous effluent on an hourly basis, and these were analyzed for glucose and lactate content. Lactate and LDH were measured using spectrophotometric assays. Glucose utilization (GU), cumulative glucose utilization (CGU), and the ratio of lactate production to glucose utilization (L/GU ratio) were used as indicators of biochemical viability (2,7,9,10) and to determine the biochemical effects of HD on the IPPSF metabolism. Hourly GU was used as the sole parameter of biochemical viability prior to dosing, and an hourly GU of less than 10 mg/hour/IPPSF or a plateau in the upward slope of CGU indicated a loss of viability in the skin flap preparation.

The vascular response of the IPPSF was characterized using the physiological parameter of vascular resistance (VR) where $VR \text{ (mmHg-min/ml)} = \text{arterial pressure/flow}$. Values are normalized to initial flap VR to minimize inherent preparation differences. Prostaglandins (PGE_2 , 6-keto $\text{PGF}_{1\alpha}$) in the venous perfusate were assayed using a column purification followed by enzyme linked immunoassay (EIA).

After 1 hr of stable perfusion, each IPPSF is treated nonoccluded with either HD (10 mg/ml) or ethanol as a control inside a flexible, plastic template (Stomahesive) having a 5 cm^2 dosing area for the time response experiments or a 7.5 cm^2 dosing area for the physiological manipulation studies. To keep the HD concentration constant, the applied dose volume was proportionally increased from 200 to 300 μl . Agent was applied using a positive displacement pipette system. The chamber temperature, airflow, and relative humidity were constant. Statistical comparisons of the viability parameters were performed by calculation of their average rate of change over the time course of perfusion (slope). Differences between treatments were analyzed by the General Linear Model procedure (Proc GLM) in SAS, and multiple comparison tests were performed using Student's (Least Significant Differences) t-test and the Ryan-Einot-Gabriel-Welsch multiple F test.

After perfusion, tissue samples were taken for light microscopy (LM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and enzyme histochemistry (EH). LM samples were fixed in 10% neutral buffered formalin. TEM samples were minced and fixed in modified Karnovsky's fixative for 24 hrs (6). The LM samples were processed routinely, embedded in paraffin, and stained with either Harris's hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). The TEM samples were routinely processed, embedded in Spurr's resin, and sections examined on a Philips 410 TEM. For SEM, tissues were fixed in 4% paraformaldehyde and 1% glutaraldehyde, processed, then mounted on aluminum stubs and sputter coated with gold-palladium, and examined on a JEOL JSM 35 CF SEM.

Tissues dissected from the flap dosing area following 1, 3, 5, and 8 hrs of HD were embedded in OCT compound and quenched in an isopentane well immersed in liquid nitrogen. Indirect immunohistochemistry was carried out using routine biotin-streptavidin methodology. Indirect immunoelectron microscopy (IEM) utilized a previously described method (17) with slight modifications and viewed on a Philips 410 TEM. For enzyme histochemical analysis six different enzymes; alkaline phosphatase (ALP), acid phosphatase (ACP), nonspecific esterase (NSE), lactate dehydrogenase (LDH), succinic dehydrogenase (SDH), and malate dehydrogenase (MDH) were assessed on the time-response flaps. Standard techniques were used as fully described in our report ADA 254419 (18). All of these enzyme histochemical techniques ACP, ALP, NSE, SDH, LDH, and MDH included paired negative controls which lacked the specific substrate. The intensity of staining was scored individually in each epidermal layer (stratum basale, stratum spinosum, stratum granulosum, and stratum corneum) as: 0 = no staining; 1 = slight; 2 = moderate; 3 = intense. Statistical analysis was performed using the Student's t test ($p < 0.05$) to compare all doses within and between the agent and control groups. The first experiment was designed to determine the time course of vesicant injury by utilizing 48 IPPSFs and dosing 10.0 mg/ml of HD at 1, 3, 5, and 8 hrs ($n=4/\text{treatment}$). A set of controls ($n=4$) at each interval was used. The second experiment was designed to study the mechanism of HD blister formation by physiological manipulation of perfusate, glucose, and pressure. Glucose of 50 mg/dl, 120

mg/dl, and 400 mg/dl, with a flow rate of 1.5 ml/min was utilized to assess the effect of glucose concentration. To assess the effect of flow flow rates of 0.5, 1.5, and 3.0 ml/min with a glucose concentration of 120 mg/dl were employed. Each set of conditions had four IPPSFs.

RESULTS

Time Response Study: VR (pressure/flow) (Fig. 1) increased over controls ($p < 0.05$) in HD-treated flaps by 2 hrs which plateaus at 5 hrs. For the ethanol controls, VR initially decreased and remained constant throughout the experiment. The fractional GUs decreased at 1-2 and 4-7 hrs, with a peak occurring at 3 hrs. (Fig. 2). This reduction in GU is more evident if the more sensitive CGU is plotted versus time (Fig. 3). An increase in VR was associated with an increase in the weight gain of an IPPSF for all treatments. LDH concentrations significantly increased over the course of a perfusion, starting at 2 hrs,

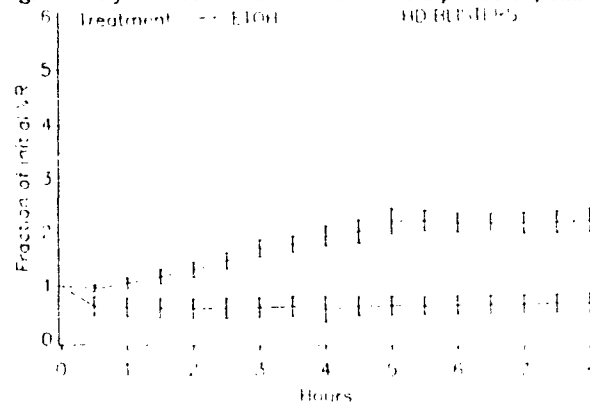


Figure 1. Graph illustrating VR of 10.0 mg/ml HD blisters compared to ethanol control.

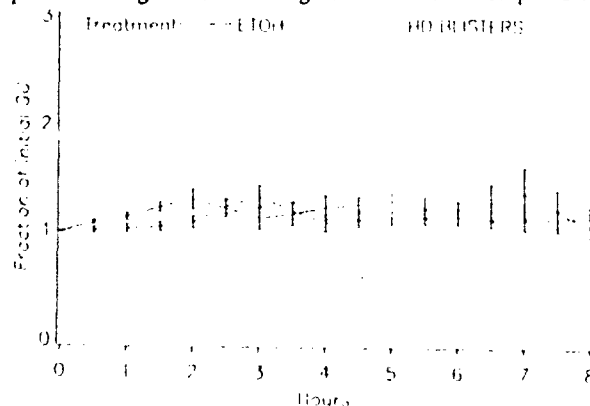


Figure 2. Graph illustrating GU of 10.0 mg/ml HD blisters compared to ethanol control.

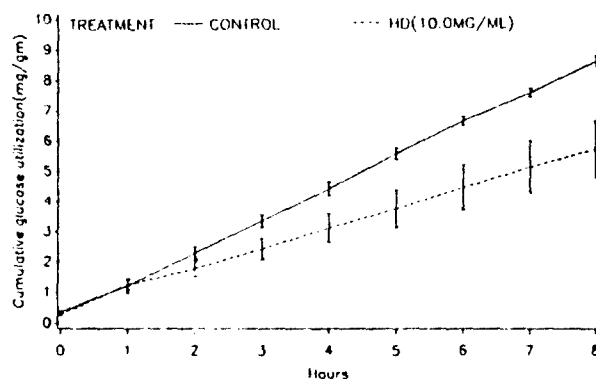


Figure 3. Graph illustrating CGU of 10.0 mg/ml HD compared to ethanol control.

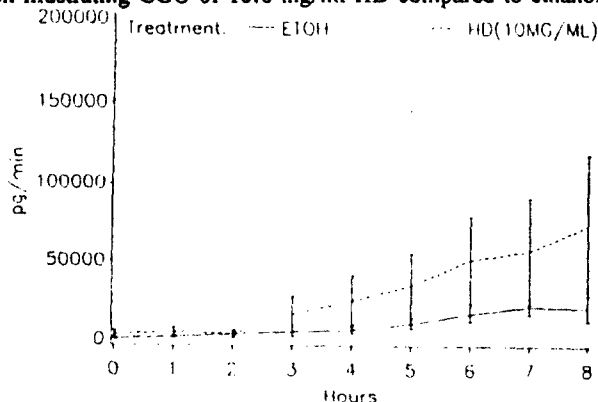


Figure 4. Graph illustrating PGE₂ flux in 10.0 mg/ml HD compared to ethanol control.

indicating cellular damage severe enough to cause a release of this enzyme into the perfusate. Lactate:glucose ratio significantly dipped at 3 hrs. Finally, HD treatment was associated with a dramatic increase in PGE₂ flux in some blistered flaps (Fig.4). 6-keto PGF_{1α} showed minimal response.

HD induced blisters are characterized by dark basal cells, pyknotic nuclei, or a preponderance of intracellular edema. No histological changes were noted at 1 hr. At 3 and 5 hrs a few dark pink dyskeratotic basal cells and intracellular edema were present. However, all of the 8 hr HD-treated IPPSFs showed a dramatic increase in the number of dyskeratotic and pyknotic basal cells (Fig.5), and epidermal-dermal separation was present. Also, this is evident by SEM (Fig.6). TEM showed no dramatic organelle damage at 1 hr. At 3 hrs, some mitochondria were so swollen that cristae were not evident, while others were elongated. Nuclear envelope separation occurred in the dark stratum basale cells (Fig.7). Mitochondria appear to have coalesced, thereby forming a large cytoplasmic vacuole within the cell. The epidermal-dermal junction as well as the upper stratum spinosum and stratum granulosum

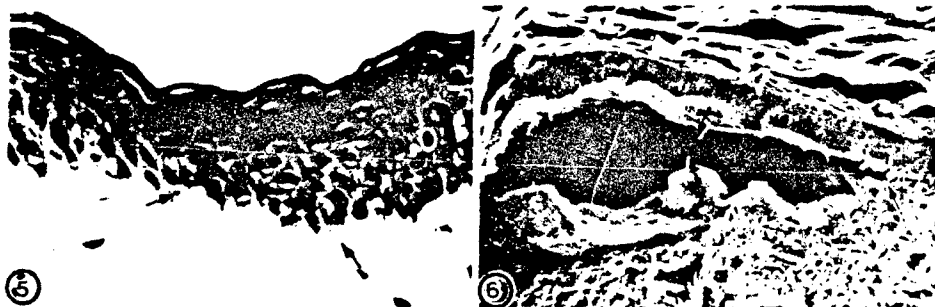


Figure 5. LM of 10.0 mg/ml HD with pyknotic stratum basale cells and basement membrane separation (arrows). x450



Figure 6. SEM of 10.0 mg/ml HD showing basement membrane separation (arrows). x200

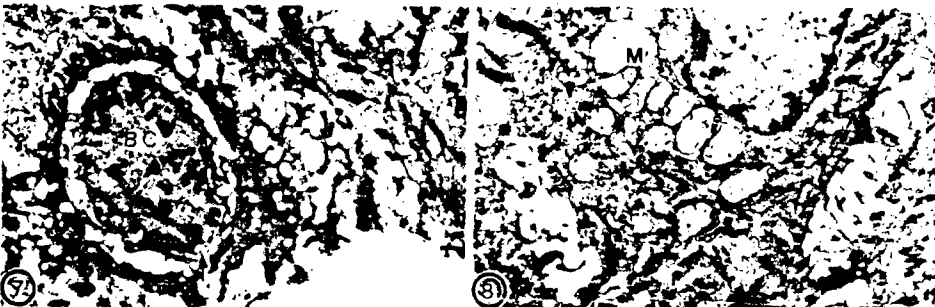


Figure 7. TEM showing nuclear envelope separation in a dark basal cell (BC) after 5 hrs of HD. x5,300

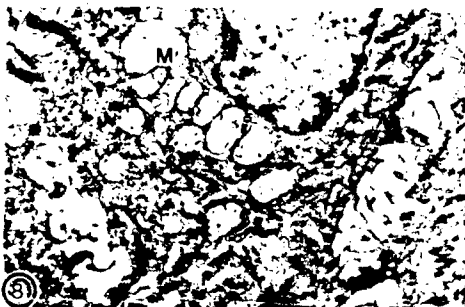


Figure 8. TEM showing coalescing of mitochondria (M) in the basale cell layer after 5 hrs of HD. x11,400

cells were normal. At 5 hrs of exposure to HD, more prominent cytoplasmic vacuoles, coalescing of mitochondria (Fig.8), and nucleolar margination and segregation were observed in the stratum basale layer. As seen with LM, a few dark basal cells were noted. At 8 hrs, there was an increase in cytoplasmic vacuolization, degenerating stratum basale cells, pyknotic basal cells, blown-out mitochondria, and nucleolar segregation. Also, epidermal-dermal separation and degenerative basal cells were present in the blistered flaps.

The statistical analyses of HD and control group enzyme histochemistry data showed a significant time relationship in the ALP, NSE, SDH and MDH histochemistries. LDH and ACP were not significant. The ALP stain was only significant in the stratum basale layer within the HD and control groups. There was a significant increase in staining at 3 hrs, which gradually decreased at 5 hrs and continued to decrease until 8 hrs. The NSE staining of the stratum corneum layer was not significant, although HD did result in a slight increase in staining at the 3, 5 and 8 hr exposures. SDH showed a significant increase in staining of the stratum spinosum layer at the 3 hr HD which decreased at 5 through 8 hrs. The controls



Figure 9. Indirect immunohistochemistry of 10.0 mg/ml HD showing type IV collagen staining (arrows). x260.

Figure 10. Indirect immunohistochemistry of 10.0 mg/ml HD showing laminin staining (arrows). x260



Figure 11. IEM of 10.0 mg/ml HD showing strong dermal staining (large arrows) and focal epidermal staining (small arrows) of laminin. x20,800

Figure 12. IEM of 10.0 mg/ml HD showing type IV collagen staining of the dermis (arrows). x23,800

demonstrated a similar and significant increase at 3 and 8 hrs, but tended to show a mild decrease at 5 hrs. Comparisons of staining between HD and controls for SDH were not significantly different. With HD, MDH activity in the stratum basale and spinosum layers increased at 3 hrs and then decreased at later time points.

Indirect immunohistochemistry and immunoelectron microscopy characterized the location of the epidermal-dermal separation. The type IV collagen stained the blister floor (dermis) exclusively (Fig.9), while laminin stained the floor and, to a lesser extent, the roof (epidermis) of the blister (Fig.10). Type IV collagen stained darker with a more discrete banding than laminin in all flaps. IEM of laminin (Fig.11) and type IV collagen (Fig.12) showed that the precise location of the epidermal-dermal cleavage occurred primarily in the upper lamina lucida.

The bullous pemphigoid antibody (BPA) exhibited strong continuous staining of the epidermal dermal junction in nonblistered flaps. In blistered flaps, BPA was limited to the basal pole of the epidermal basal cells.

Physiological manipulations: Analysis of all ethanol- and HD-treated IPPSFs with altered glucose (50, 120, 400 mg/dl) and altered flow (0.5, 1.5, 3.0 ml/min) yielded the following results. High perfusate flow and low glucose tend to cause blister formation in ethanol-treated flaps. However, these blisters were not associated with the presence of dark basal cells. As expected, all HD-treated flaps produced typical appearing blisters. The major exception to this was in the high glucose (400 mg/dl) group in which only one epidermal-dermal separation was noted. This group had dark basal cells and in common with the control, intracellular edema. Glucose level was a major determinant of blister formation in the IPPSF. If glucose was high, blisters did not form with HD treatment, even though dark basal cells and intracellular edema were present. In contrast, low glucose in ethanol-treated flaps potentiated blister formation in the absence of dark basal cells.

TEM of the low glucose, normal flow (50 mg/ml, 1.5 ml/min) revealed epidermal-dermal separation and ruptured mitochondria with undistinguishable cristae. Damaged dark stratum basale cells and large cytoplasmic vacuoles were seen in some of the HD-treated flaps. High glucose, normal flow (400 mg/dl, 1.5 ml/min) revealed an increased number of dark basal cells only in the HD-treated flaps and not in the ethanol controls. Normal glucose and low flow (120 mg/dl, 0.5 ml/min) also showed a high incidence of dark basal cells in the HD-treated flaps. Dilated endoplasmic reticulum in the lower stratum spinosum cells and pyknotic basal cells were sometimes present. The upper stratum spinosum, granulosum, and corneum cell layers appeared normal. The HD-treated flaps with normal glucose and normal flow (120 mg/dl, 1.5 ml/min) had similar organelle changes as previously described. Flaps treated with normal glucose and high flow (120 mg/dl, 3.0 ml/min) revealed numerous dark basal cells, nucleolar segregation, vacuolization, damaged mitochondria, basement membrane separation and degenerative stratum basale cells when compared to controls.

When the flow rate was held constant at the normal value of 1.5 ml/min and glucose concentration was changed to 50, 120 (normal) and 400 mg/dl, an increase in VR was only seen in those IPPSF groups associated with the formation of blisters (ethanol at 50 mg/dl, HD at 50 and 120 mg/dl). When glucose concentration was held constant at the normal value of 120 mg/dl and perfusate flow was changed to 0.5, 1.5 (normal), and 3.0 ml/min, very little change in VR was noted in the ethanol-treated groups. In HD treated groups, both high and low flow seemed to blunt the normal increase seen in VR when blisters were induced. When CGU is examined, GU's in the 1.5 and 3.0 ml/min ethanol-treated groups were not different from one another, but both were greater than in the 0.5 ml/min group. In the HD-treated flaps, there was a direct relationship between CGU and perfusate flow.

DISCUSSION

Immunohistochemistry of HD-treated IPPSFs demonstrated that the epidermal-dermal separation occurred at the level of the lamina lucida. Type IV collagen stained the dermal floor, while laminin stained the blister floor and, to a lesser extent, the roof. Bullous pemphigoid antigen showed staining of the hemidesmosomes of the basal cell membrane. In

the IPPSF, the formation of a blister, regardless of etiology, is marked by an increase in VR over the course of an experiment. An increase in VR was correlated to an increase in weight gain over the course of an experiment. This is presumably due to edema, an hypothesis supported by the increased PGE_2 flux observed. A peak in GU and release of LDH into perfusate occurred at 3 hrs in HD flaps. The lactate:glucose ratio decreased at 3 hrs after HD and then returned to normal.

Morphologically, no changes were evident with HD exposure at 1 hr. However by 3 hrs, significant intracellular changes were noted with LM and TEM for HD. The HD flaps contained some dyskeratotic cells. TEM of HD flaps showed basal cells with swollen mitochondria, some of which coalesced into larger vacuoles, thereby pushing the nucleus to one side. There was nuclear envelope separation and some degenerative basal cells. Importantly, there was no epidermal-dermal separation at this early time. Enzyme histochemistry also showed the most dramatic changes at this 3 hr time point. Both ALP and to a slighter extent MDH peaked at 3 hrs and then declined at the later time points. NSE also started to increase at 3 hrs. By 5 hrs, LM of HD flaps appeared similar to the 3 hr. TEM was characterized by nucleolar margination and nucleolar segregation, larger intracellular vacuoles, and the presence of more dark basal cells. Again, these changes were not associated with epidermal-dermal separation. The 8 hr flaps were similar to those described in previous studies.

The most significant finding of the time-response study was that HD-induced cellular damage occurred **before** any evidence of epidermal-dermal separation. These changes were characterized by intracellular edema, and by nucleolar and mitochondrial changes. It is particularly intriguing that increases in GU occurred at 3 hrs in HD flaps, the same time point when initial cellular changes were seen morphologically and enzyme levels changed on histochemistry. These changes were present even in the absence of epidermal-dermal separation, suggesting that they may be critical early markers of impending vesication.

The physiological experiments provided supporting evidence for the importance of cellular changes and the central role of glucose metabolism in HD-induced vesication. As expected, HD treatment of all flaps produced dark basal cells adjacent to areas of microvesication. Intracellular edema was also present. All HD flaps blistered **except** for those with a high (400 mg/dl) glucose in the perfusate. In these flaps, cellular changes such as intracellular edema and dark basal cells were present, GU increased, and VR was unchanged. In contrast, low glucose (50 mg/dl) perfusate induced blisters in the absence of HD treatment and the VR of this group was elevated. In both HD and ethanol groups, changes in glucose concentration were directly correlated to changes in GU, suggesting that in this system, GU was a function of availability. VR did not change as a function of GU, nor did the lactate:glucose ratio. The effect of altering perfusate flow was less dramatic. Change in perfusate flow did not change VR. However, high perfusate flow prevented the normal increase in VR associated with blister formation. There was a tendency towards spontaneous blistering at normal or elevated flow rates; however, cellular changes were not present. When perfusate flow decreased to 0.5 ml/min, GU was decreased. No differences were noted between 1.5 and 3.0 ml/min.

In summary, it is evident that the pathogenesis of HD-induced vesication is a multifactorial process associated with DNA damage and general cytotoxicity. The IPPSF

appears to be a relevant model with which to study this process, since the morphological appearance and the biochemical and physiologic effects are very similar to that reported in humans. Importantly, gross blisters also form in this in vitro model, a unique attribute of the IPPSF.

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